Applications of 'hot' and 'cold' bis(thiosemicarbazonato) metal complexes in multimodal imaging

Fernando Cortezon-Tamarit, Sophia Sarpaki, David G. Calatayud, Vincenzo Mirabello and Sofia I. Pascu*

Department of Chemistry, University of Bath, Claverton Down, Bath, BA2 7AY, UK
Email: s.pascu@bath.ac.uk

ABSTRACT: The applications of coordination chemistry to molecular imaging has become a matter of intense research over the past 10 years. In particular, the applications of bis(thiosemicarbazonato) metal complexes in molecular imaging have mainly been focused on compounds with aliphatic backbones due to the *in vivo* imaging success of hypoxic tumors with PET (Positron Emission Tomography) using ^{64}CuATSM [copper (diacetyl-bis(N4-methylthiosemicarbazone))]. This compound entered clinical trials in US and UK during the first decade of the 21st century for imaging hypoxia in head and neck tumors. The replacement of the ligand backbone to aromatic groups, coupled with the exocyclic N's functionalization during the synthesis of bis(thiosemicarbazones) opens the possibility to use the corresponding metal complexes as multimodal imaging agents of use both in *in vitro* for optical detection, and *in vivo* when radiolabeled with several different metallic species. The greater kinetic stability of acenaphthenequinone bis(thiosemicarbazonato) metal complexes with respect to that of the corresponding aliphatic ATSM complexes allowed the stabilization of a number of imaging probes, with especial interest in 'cold' and 'hot' Cu(II) and Ga(III) derivatives for PET applications and ^{111}In(III) derivatives for SPECT (Single-Photon Emission Computed Tomography) applications, whilst Zn(II) derivatives display optical imaging properties in cells, with enhanced fluorescence emission and lifetime with respect to the free ligands. Preliminary studies have shown that gallium-based acenaphthenequinone bis(thiosemicarbazonato) complexes are also hypoxia selective *in vitro,* thus increasing the interest in them as new generation imaging agents for *in vitro* and *in vivo* applications.

Introduction

There has been some significant interest in the use of nitrogen/ sulfur/oxygen (N/S/O) donors to generate metal complexes with high kinetic stability in aqueous environments.[1–4] The thiosemicarbazonato motif has been known since the early 1900 and bis(thiosemicarbazones) (BTSC) were described as a way to functionalize and isolate diketones.[5] However, their study was systematized and their properties as metal chelators understood only from the 1950s.[6] The great interest in these compounds and their metal complexes was mainly motivated by their biological activity. They are known to have properties as antibacterial,[7],[8] antifungal,[9],[10] antiviral,[11] or antineoplastic[12],[13]. Through the pioneering work of Fujibayashi,[14],[15] Blower,[16] Lewis[15],[17] and Dilworth[18] there has also been a great interest in the application of bis(thiosemicarbazonato) metal complexes as molecular imaging agents, especially due to the hypoxia selectivity of some of these derivatives. CuATSM has certainly been the most studied bis(thiosemicarbazonato) metal complex so far. It has demonstrated its efficacy as an imaging probe to delineate hypoxia in tumors and cardiac ischemia.[14,19–21] It was first used in clinical trials in 2000[22] and further clinical studies had provided useful information in patients with lung,[22],[23] cervical[21],[24] and

Sofia Pascu is a Reader in Inorganic Chemistry at the University of Bath, and ERC Consolidator grantee (2014–2019). She is an Academic Visitor to the Oxford Siemens Molecular Imaging Laboratory, University of Oxford (2007–2015). She has an extensive track record in 'metals in medicine' as well as in the development of new imaging agents based on small molecules and nanomaterials. She was the McCamley Memorial Lecturer, University of York 2007, the Draper's JRF in Sciences of St Anne's College, University of Oxford (2005–2007) and the Dervorguilla Scholar of Balliol College, University of Oxford as a DPhil student (1998–2002).

Fernando Cortezon-Tamarit is an Early Stage Researcher into the Marie Curie ITN PROSENSE. His project is based on the functionalization of small molecules and carbon nanotubes for the imaging of prostate cancer cells.

Sophia Sarpaki is a Postgraduate Student in the Department of Chemistry at the University of Bath since October 2014 funded by the O2SENSE grant of the ERC. Her project is focused on the synthesis of multimodal imaging agents towards hypoxia selective inhibitors.

David G. Calatayud is a Postdoctoral Research Associate in the Department of Chemistry at the University of Bath since October 2014 funded by the O2SENSE ERC Consolidator grant. His current research is focused on 'smart' all-in-one multimodal imaging probes as novel synthetic platform systems for personalized diagnosis and treatment of diseases such as cancer.

Vincenzo Mirabello graduated in Pharmaceutical Chemistry from the University of Palermo in 2008. In 2009 he moved to ICCOM – CNR, National Research Council of Italy, in Florence as a PhD student and later as a postdoctoral fellow under the supervision of M. Peruzzini and L. Gonsalvi. In Florence, he studied the activation and transformation of white phosphorus by late transition metals and its dynamic behavior in solution and solid state via NMR spectroscopy. In February 2013, he joined Sofia I. Pascu's group at the University of Bath (UK) as a Research Associate developing multimodality imaging probes for hypoxia sensing. His interest focus on NMR spectroscopy, dynamic supramolecular chemistry, nanotechnologies and the use of transition metals in medicinal chemistry.

rectal[25] cancers. A number of reviews about thiosemicarbazones have appeared in the literature over the recent years. The first example, reported by Livingstone *et al*.,[26] included thiosemicarbazones as part of a broader work in N/S donor ligands. In subsequent cases, the works have been centered on the thiosemicarbazide and thiosemicarbazone ligands, looking mainly at the coordination chemistry[27] with an exhaustive overview by Lobana *et al.* in 2009.[28] Specific reviews applied to the role of bis(thiosemicarbazonato) complexes in imaging had also appeared in the recent literature[29] along with structural studies and application of copper(II) derivatives as drugs[30] and radiopharmaceuticals.[31]

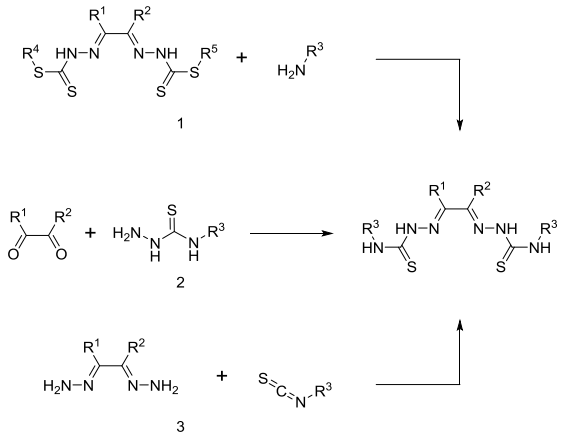
As mentioned above, the main current applications of bis(thiosemicarbazonato) complexes have been focused on PET with ^{64}Cu. However, in this context, other metals as zinc(II), gallium(III) or indium(III) have also been explored.[3] The broadening of the scope of metals, along with the substitution of the thiosemicarbazide and the change in the backbone has provided fluorescent bis(thiosemicarbazonato) complexes and allowed their use in multimodal imaging, i.e. as imaging agents in more than one technique.[32] Bis(thiosemicarbazonato) complexes have so far been used in pre-clinical research of reference to PET, SPECT and confocal microscopy. [1],[2],[4]

The aim of this review is to provide a personal account of the general background on the use of bis(thiosemicarbazonato) metal complexes in molecular imaging, which is central to our recent work, with a particular emphasis on their role in recent advances in molecular imaging. Over the past decade, we have sustained work forwards a long-lasting interest in understanding the structure-function relationships underlining the potential of bis(thiosemicarbazonato) metal complexes for applications in multimodal imaging involving PET/SPECT/optical modalities. The molecular design and the selectivity of this class of compounds for the evaluation of hypoxia will be highlighted herein.

Synthesis

Synthesis of ligands

To date, the synthesis of bis(thiosemicarbazone) ligands has been mainly carried out by functionalization of a 1,2-diketone with a thiosemicarbazide forming the iminic bond (Scheme 1, 2) by heating both components in an alcoholic solution in the presence of an acid catalyst.[33] The syntheses are generally successful and proceed in good yields, but alongside the desired reaction pathways, a number of side reactions leading to cyclization



Scheme 1 Different synthetic routes to aliphatic bis(thiosemicarbazones)

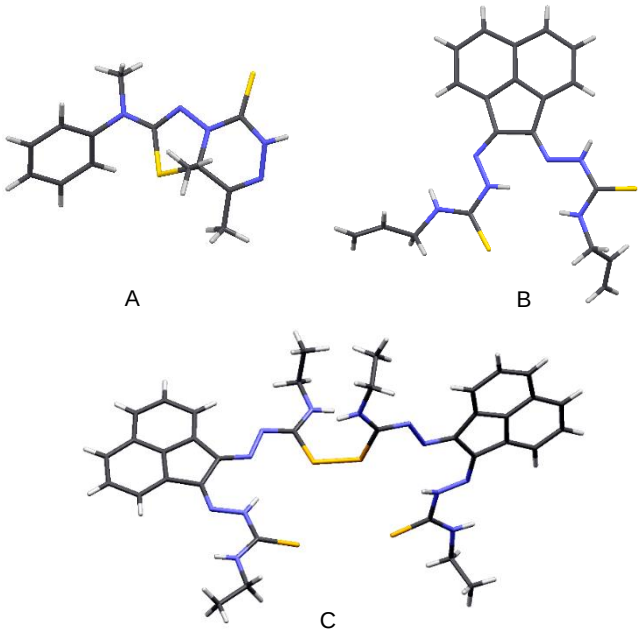


Figure 1 Molecular structures of A) bicyclic subproduct observed during the synthesis of aliphatic bis(thiosemicarbazones[34]; B) bis(4-allyl-3-thiosemicarbazone) acenaphthenequinone ligand; C) bis(4-ethyl-3-thiosemicarbazone) acenaphthenequinone derivative presenting a disulfide bridge[1]

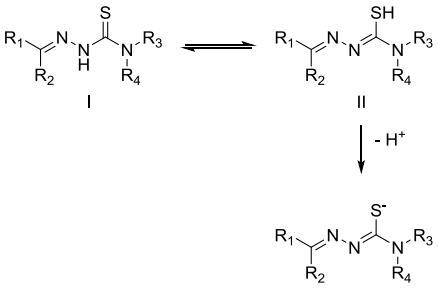
products or dimers have also been observed to occur (Figure 1, A, C).[34],[35]

Several other synthetic strategies were developed over the last decades to achieve thiosemicarbazones as using hydrazones, oximes or dithiocarbazides as precursors.[33] In 2006 a compendium of the available methods of synthesis and potential problems in the synthesis of bis(thiosemicarbazones) as ligands for molecular imaging was reported by Dilworth *et al.*[35] The majority of bis(thiosemicarbazones) reported in the literature are derived from 1,2-diketones. However, the use of 1,3-,[36] 1,4-[36],[37] or 1,5-[38] diones have been reported. The most studied precursors are perhaps 2,3-butanedione, pyruvaldehyde and glyoxal which were found to lead to the formation of most of the members in the family ATSM ligands and their complexes.

The synthesis of symmetrical bis(thiosemicarbazones) is generally less challenging. However, successful examples of unsymmetrical BTSC ligand derivatives can also be obtained.[35] Unsymmetrical vicinal dicarbonyl compounds can be used as

Table 1. Metals reported to form thiosemicarbazones complexes, by Group in Periodic Table[28]

5	6	7	8	9	10	11	12	13	14	15
								Al		
V	Cr	Mn	Fe	Co	Ni	Cu	Zn	Ga		
	Mo	Tc	Ru	Rh	Pd	Ag	Cd	In	Sn	
	W	Re			Pt	Au	Hg	Tl	Pb	Bi
U										



Scheme 2 Tautomeric equilibrium in thiosemicarbazones

precursors of the reaction. However, they can easily lead to mixtures of components that are complicated to purify.[39]

A stepwise condensation of a 1,2-dicarbonyl compound with two different thiosemicarbazides is the most exploited strategy although it was reported that the reaction conditions need to be controlled carefully, as even small variations in time or temperature can result in disproportionation of the product giving a statistical mixture of products.[35],[40]

This behavior could be attributed to the reversible character of the imine-enamine bond[41] coupled with the capability of thiol groups to undergo, in the presence of O_2, an exchange process leading to the formation of disulfide S-S bonds. These features specific to thiosemicarbazones might result in a dynamic correlated exchange leading to complex mixtures under thermodynamic control. The production of dynamic combinatorial libraries involving the thiol-disulfide exchange, imines and hydrazones have been well studied[42] and our current experimental observations point towards the fact that a similar reactivity is likely to occur for the bis(thiosemicarbazone) species studied recently. The dynamic combinatorial exchanges are influenced by the variable pH, water/air presence and solvents such as DMSO: all these conditions can affect the synthesis of all mono- and bis(thiosemicarbazones) and the behavior of the resulting metal complexes species en route for imaging applications.

Coordination Chemistry

Thiosemicarbazones are incredibly versatile ligands. In solution phase, they have been found in a tautomeric equilibrium between

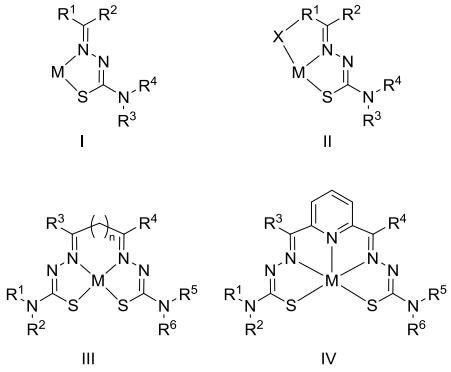


Figure 2 Common coordination modes of mono and bis(thiosemicarbazone) ligands to the metal

the thione and thiol forms (Scheme 2). Therefore, they can bind to the metal in neutral form as a thiol or anionic form through the iminic nitrogen and sulfur atom when the thiol proton is lost.

These ligands provide an anionic S^{2-} ligand ready to bind to the metal without the tendency of thiolate to form disulfide bridges in the presence of metal atoms.[43] They have been known to act as chelators of a wide variety of metals including transition metals, main group metals or semimetals as seen in Table 1. A large number of examples can be found in the reviews mentioned in the introduction.[27–29]

The corresponding 3d metal complexes are usually obtained by refluxing the ligand and the metal acetate, halide, sulfate, etc., although the reaction can also be carried out under room temperature conditions.[28]

The binding of mono(thiosemicarbazones) occurs normally through the N/S atoms acting as bidentate ligands. However, they can also act as tridentate ligands if any of the substituents has a donor atom and it is involved in the coordination to the metal (Figure 2, II). Other, coordination possibilities have been described, as monodentate through the S atom or bidentate and tridentate with the S atom bridging different metals but these examples will not be discussed here. Bis(thiosemicarbazones) can also bind in neutral and anionic form by the N and S atoms through both arms acting as tetradentate ligands. The resulting complexes have usually the form of III or IV in Figure 2. The latter includes the participation of a donor atom in the backbone group between the imine moieties in the coordination to the metal.

In this work, the overview of the coordination chemistry is mainly focused on tetradentate bis(thiosemicarbazones) chelating the metals of interest in imaging and their precursors (i.e. Zn(II), Cu(II), Ga(III), and In(III)).

The analysis of the X-ray structures for a family of acenaphthenequinone bis(4-methyl-3-thiosemicarbazonato) metal complexes is reported in the following sections. Molecular parameters such as bond distances, angles and the resulting geometries of novel zinc(II), copper(II), nickel(II), gallium(III) and indium(III) complexes are discussed (vide infra).

Multimodal imaging

The use of different imaging techniques in order to have better visualization of a pathogenic condition either *in vitro* and/or *in vivo* became an established field over the past 10 years. PET, SPECT and confocal fluorescence are the main biomedical imaging techniques widely used for imaging tumours either *in vivo* or *in vitro*. The first two are based on emission tomography (ET) principles. ET utilizes radioactive elements (radiotracers) to permit the visualization of the body's anatomy and can depict the spatial distribution of different physiological processes such as glucose metabolism thus facilitating the detection of the relevant diseases (tumors, etc.).[44] However, they are distinguishable because of the type of the radioisotope each one uses. As indicated by their names, PET's isotopes of choice are positron emitters, whereas SPECT uses single photon emitters. The third

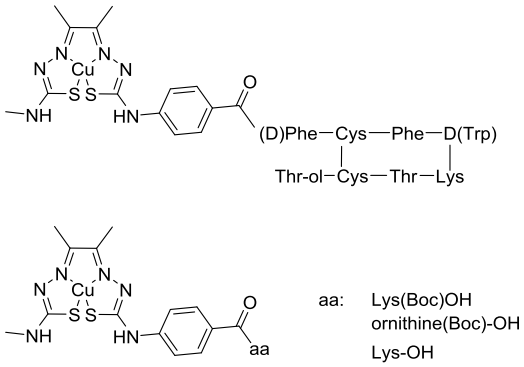


Figure 3 Structures of peptide-functionalized copper complexes of bis(thiosemicarbazones)

method mentioned above is an imaging technique which uses the excitation states of fluorescent molecules to image specific biological systems. All three techniques could potentially be used in combination resulting in better images of the same target. The combination of PET and MRI has also generated a great interest over the last few years. This topic, however, will not be discussed herein as it has made the topic of several different reviews and reference books.[45-47]

The use of bis(thiosemicarbazonato) metal complexes has been normally limited to PET with ^{64}Cu or ^{68}Ga aliphatic complexes. Some research has been conducted on the dual labelling of ATSM ligands with ^{64}Cu and other radionuclides as ^{123}I[48] or ^{18}F[49] in an attempt to understand the biological fate of copper *in vivo* and clarify the mechanism of hypoxia selectivity. In addition to the dual labelling of bis(thiosemicarbazonato) metal complexes to

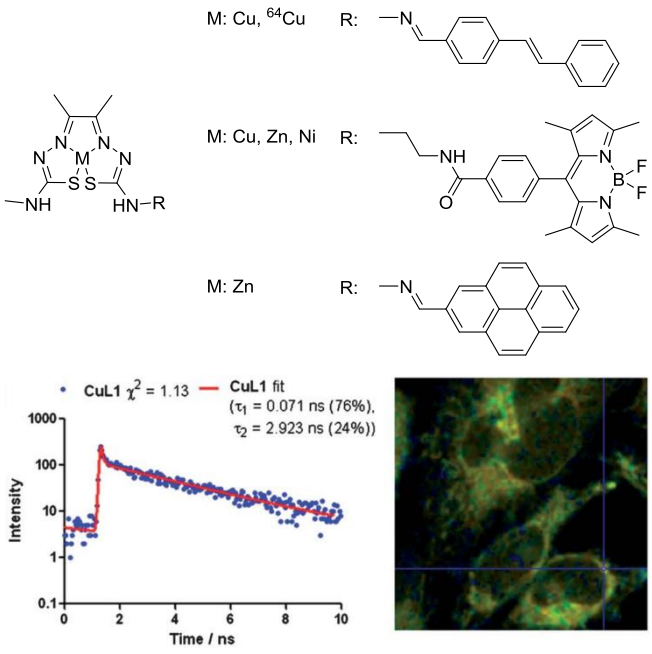


Figure 4 Structures of ATSM complexes incorporating aliphatic backbones studied by fluorescence microscopy (top) and fluorescence lifetime maps and sample point decay curves of a CuATSM-BODIPY complex in HeLa cells after 20 min incubation (bottom). Adapted from Ref. [4] with permission from The Royal Society of Chemistry

study their use as multimodal imaging agents, the derivatization with biologically active molecules has also been explored.
Dilworth *et al.* prepared a copper complex with one of the ligands including a carboxyphenyl thiosemicarbazide. The carboxylic acid functionality was used to attach different peptides including octreotide, a somatostatin analog with improved pharmacological properties (Figure 3).[50],[51] However, the first *in vitro* and *in vivo* study of a functionalized bis(thiosemicarbazone) involved the functionalization of a thiosemicarbazide with an aromatic group bearing different functionalities as hydroxyl, nitro or even a nitroimidazol moiety. Some of the examples proved to be hypoxia selective *in vitro* and *in vivo* although with a high liver uptake, pointing out to the necessity of opening new directions into the field of functionalization of these compounds, to bypass limitations in liver biodistribution.[52] A similar strategy was used by Donnelly *et al.* to synthesize a copper bis(thiosemicarbazone) complex with bombesin, a targeting peptide towards receptors overexpressed in prostate cancer cells. In this case, the radiolabelling with ^{64}Cu is reported but not *in vitro* or *in vivo* studies were carried out.[53] The same group explored the preparation of copper radiopharmaceuticals using bombesin as a target but changing the chelating system to macrobicyclic cages.[54]

Dilworth *et al.* continued the study of bifunctional chelators by functionalization of a NH_2 tagged CuATSM variant with aliphatic and aromatic carboxylate linkers.[55] The prepared derivatives were coupled to biomolecules, namely biotin and bombesin, the latter by reacting the metallic complex with the peptide during the solid phase synthesis. The compounds were radiolabeled at room temperature with ^{64}Cu and ^{99m}Tc showing applicability to PET and SPECT studies. Furthermore, the *in vitro* binding to PC-3 cells and *in vivo* localization in mice was evaluated.

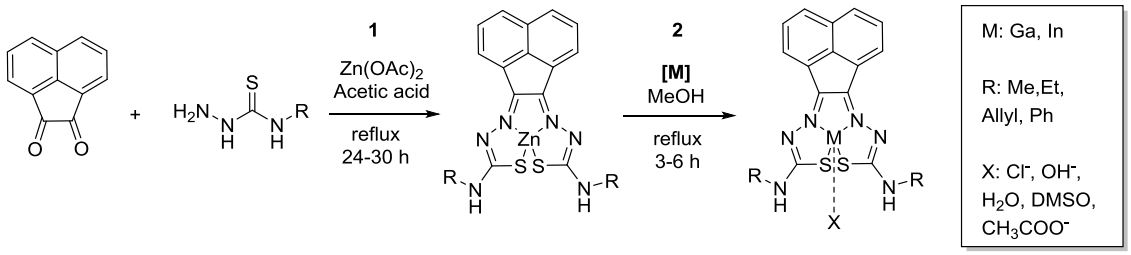
The study of aliphatic bis(thiosemicarbazonato) metal complexes using optical techniques has been difficult due to the lack of fluorescence of the complex or the thiosemicarbazone substituents. Despite these difficulties, ATSM-like bis(thiosemicarbazone) complexes had been used to monitor the *in vitro* uptake of its metal adducts by fluorescence imaging. Dilworth *et al.* discovered a fluorescent ZnATSM derivative with modified side chains and studied the uptake in different cell lines by fluorescence microscopy. This work proved that the uptake of the complex was strongly cell line dependent.[56] Previous studies also established that fluorescence was driven by an intraligand excitation.[57] However, the fluorescence intensity of the aforementioned derivatives was weak.

In fact, excluding the latter example, no fluorescence microscopy study of the behavior of these compounds *in vitro* was possible unless a fluorophore was attached to the bis(thiosemicarbazonato) core thus modifying their original properties.

Groups acting as a fluorophore have been attached to ATSM-like complexes in order to study their properties in cells (Figure 4). The use of pyrene with Cu(II) and Zn(II) complexes has been evaluated and the uptake in HeLa cells investigated. Pyrene is known to act as DNA intercalator.[58] Therefore, such planar system, was deliberately selected in this work in order to prepare a hypoxia selective compound that targeted nuclear DNA. However, the complexes did not present nuclear uptake and their uptake was not enhanced under hypoxia.[59] A similar approach was followed by Donnelly *et al.* with a different purpose. In the same manner as cited above, they appended a stilbene group to one of the arms of a Cu(II) complex that, excluding this group, had the structure of CuATSM. The stilbene group provides a target to amyloid-β plaques deposited in the brain tissue under Alzheimer or dementia diseases.[60] The stilbene functionality also kept its original fluorescence when the complex was formed despite the presence of Cu(II). Therefore, its interaction with amyloid-β plaques in human brain sections was studied by epi-fluorescence microscopy.[61] Recently, examples of fluorescent thiosemicarbazonato complexes derived from pyrene and anthracene have also been prepared and tested *in vitro*.[62],[63] However, in both cases, the thiosemicarbazone acted as a bidentate ligand. The copper complexes derived from the anthracene ligand were studied as chemoterapeutic agents for cervical cancer[62] while the rhodium and iridium pyrene derivatized half-sandwich complexes were found to interact with DNA and presented some interest against lung and breast cancer cell lines[63].

As mentioned above, the possibility of tagging the ATSM core with a chromophore suitable for fluorescence *in vitro* had drawn the attention of several research groups over the last years. Dilworth, Pascu and collaborators reported a novel approach to evaluate the stability of bis(thiosemicarbazonato) complexes *in vitro*.[4] This objective was achieved by linking a BODIPY fluorophore to the CuATSM core. BODIPYs are known to be bright fluorophores with high quantum yields and good stability in biological media.[64] Interestingly, this was the first case of application of Fluorescence Lifetime Imaging Microscopy (FLIM) coupled to confocal laser scanning microscopy to the study of ATSM complexes. The results of these experiments are discussed on the following sections.

The synthesis of bis(thiosemicarbazones) from an aromatic diketone, like acenaphthenequinone, would resolve the problem of the attachment of a fluorophore to the complex as the backbone would be intrinsically fluorescent. Examples of mono(thiosemicarbazones) synthesized from acenaphthenequinone have been reported in literature since the 1950s when the efficacy of thiosemicarbazide derivatives in the treatment of tuberculosis was discovered.[65] Other examples followed, including the formation of acenaphthenequinone mono(thiosemicarbazonato) complexes with several metals (Mg(II), Co(II), Ni(II), Cu(II), Zn(II), Cd(II)) involved in physicochemical studies[66] and Ru(III) and Pt(IV) for analytical purposes.[67] It is worth noting that mono(substituted) ligands presented antiproliferation activity. Iron, nickel, copper and zinc mono(thiosemicarbazone) adducts were studied by Fava *et al.* These studies suggested that such ligands and selected complexes presented inhibition of cell proliferation and DMSO-induced differentiation.[8] This research can certainly be considered an important achievement in the chemistry of thiosemicarbazones and its metal adducts by applying a different non-aliphatic backbone. However, it did not explore the capability of this class of ligands to act as tetradentate ligands and performance as fluorescent probes.



Scheme 3 Synthesis route to acenaphthenequinone bis(thiosemicarbazonato) complexes. The best way to obtain complexes with metals different than Zn (i.e. Cu, Ga, Ni, In) was via transmetalation from the corresponding Zn derivative

It was not until 2007 that Pascu *et al.*[68] reported the first metal complexes of Zn(II) and Cu(II) based on an acenaphthenequinone bis(thiosemicarbazone) ligands. The intrinsic fluorescence of the acenaphthene group in the backbone of the metal complex allowed the *in vitro* study of the uptake in different cancer cells. In this case, the zinc(II) complex can be obtained in a template process by refluxing acenaphthenequinone and an excess of thiosemicarbazide in the presence of zinc acetate (Scheme 3, step 1).

It was found that the most efficient strategy to arrive to other metal complexes as nickel(II), copper(II), gallium(III) or indium(III) was by transmetalation of the zinc(II) derivative (Scheme 3, step 2).[2],[3] The synthesis of acenaphthenequinone mono and bis(thiosemicarbazone) ligands has also been reported. They can be obtained via conventional heating refluxing acenaphthenequinone with the thiosemicarbazide in ethanol in the presence of hydrochloric acid.

However, our recent work reported a new procedure which applied microwave radiation to obtain these derivatives: we found that the synthesis was faster and superior yields were obtained whilst opening up the field of sustainability in the production of 'hot' as well as 'cold' molecular imaging agents.[1]

The viability of the zinc(II) bis(4-allyl-3-thiosemicarbazonato) complex to carry out PET experiments was assessed by the radiolabeling of the cited complex with ^{64}Cu. The reaction proceeded in 30 min using $^{64}Cu(OAc)_2$ and was analyzed by High Performance Liquid Chromatography (HPLC) integrated with a UV and radioactivity detectors. One of the advantages of the use of the transmetalation reaction to obtain the hot complex is that the cold zinc(II) complex could be prepared, purified and characterized with everyday laboratory facilities.

Further studies explored novel substituents of thiosemicarbazone such as exocyclic groups and Zn(II) complexation. The uptake in cellular cancer cells was studied as well as the cytotoxicity towards some standard cancerous cell lines, that was found to be comparable to that of cisplatin.[69]

Furthermore the systematic study of the synthesis and properties of bis(thiosemicarbazonato) acenaphthenequinone complexes was reported in the paper published by Pascu *et al.* in 2010. In this work, the coordination of zinc(II), nickel(II) and copper(II) with ethyl and methyl thiosemicarbazone was investigated.[2]
In the first two cases, the complexes have been synthesized in a one-pot template process. Acenaphthenequinone and an excess

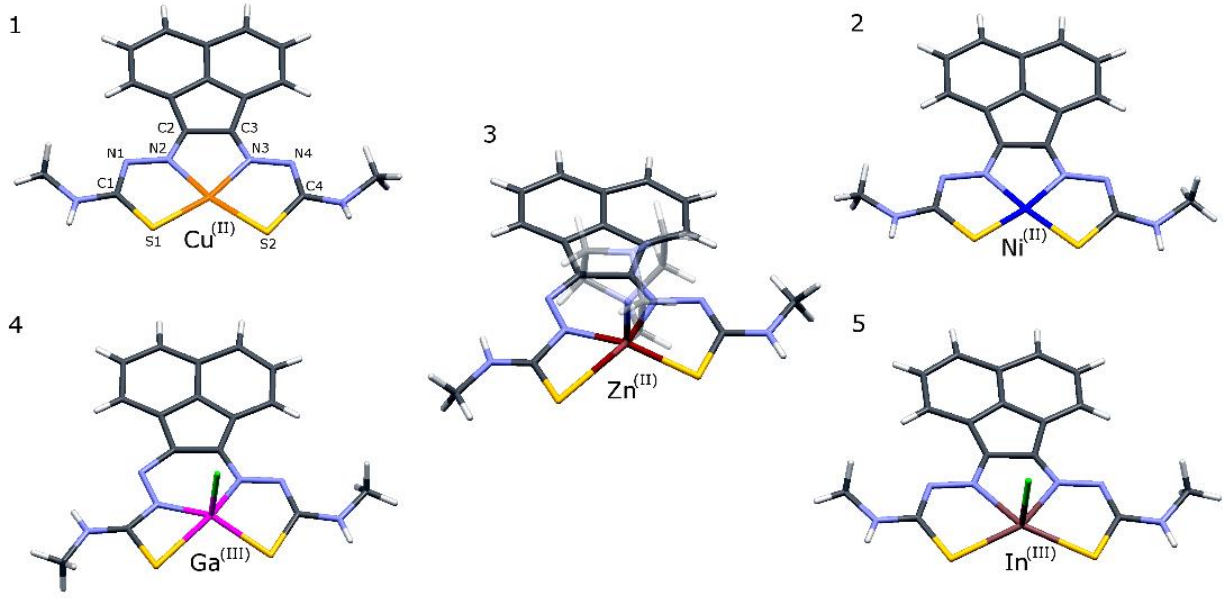


Figure 5 Molecular structures of different bis(4-methyl-3-thiosemicarbazonato) acenaphthenequinone metal complexes. Color codes: S: yellow, N: blue, C: dark grey. H: white

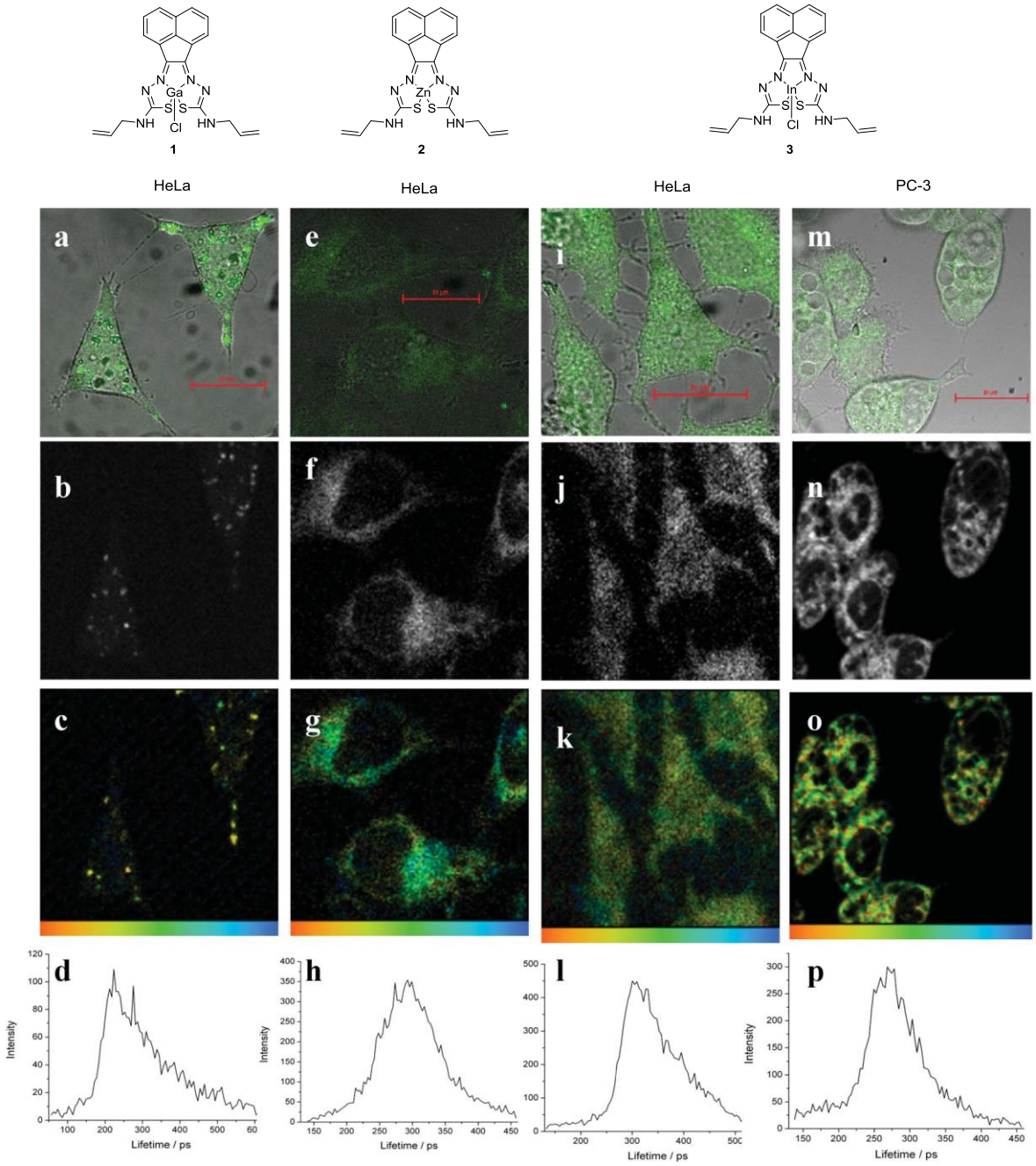


Figure 6 Confocal microscopy and fluorescence lifetime imaging of metal complexes **1**-**3** in HeLa cells and **3** in PC-3 cells. All complexes were added in 50 µM concentration and imaged after 1 h incubation. a) e) i) m) 1-Photon fluorescence emission – overlay of green and bright field channels (λ_{ex} 488 nm); b) f) j) n) 2-photon fluorescence imaging – intensity image (λ_{ex} 910nm); c) g) k) o) 2-photon fluorescence lifetime imaging map (λ_{ex} 910 nm) and d) h) l) p) corresponding lifetime distribution plot. Scalebar: 20 µm. Adapted from Ref. [3] with permission from The Royal Society of Chemistry.

of thiosemicarbazide generate the corresponding bis(thiosemicarbazone) in the presence of the metal the coordination of which acts as a driving force for the reaction. However in the case of the copper(II) complex, this strategy failed and the complex was only obtained by Zn(II)/Cu(II) transmetalation in the presence of $Cu(OAc)_2$ (Scheme 3). The hot transmetalated ^{64}Cu derivative was obtained after a 20 min reaction. The cold complexes were characterized by single crystal X-ray diffraction and EPR, as well as by cyclic voltammetry techniques.

X-ray structures of the complexes were obtained (Figure 5) and a coordination equilibrium was observed in addition to the E/Z geometric equilibrium present when the substituent of the thiosemicarbazone was an allyl group (Scheme 4).

The geometry formed in the case of copper(II) and nickel(II) is square planar. The coordination mode of the ligand leads to the formation of three five-member chelate rings that confer high stability to the complex. The ligand skeleton can be considered planar with a maximum deviation from the least-square plane of 0.040 Å for Cu and 0.044 Å for the Ni (Figure 5 and Table 2). In the case of other metals as zinc(II), galium(III) and indium(III) the

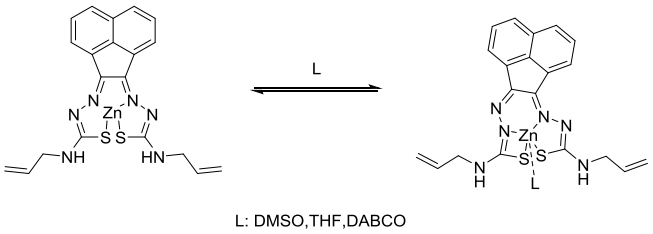
Table 2 Selected bond distances and angles for the bis(thiosemicarbazonato) metal complexes

	1	2	3	4	5
C2-C3 (Å)	1.470(6)	1.477(5)	1.495(6)	1.468(12)	1.490(2)
C2-N2 (Å)	1.307(6)	1.299(3)	1.284(6)	1.370(2)	1.306(19)
C3-N3 (Å)	1.309(6)	1.299(3)	1.314(6)	1.356(9)	1.320(19)
N2-C2-C3 (°)	116.2(4)	113.8(15)	134.3(4)	140.1(9)	117.3(15)
C2-C3-N3 (°)	116.1(4)	113.8(15)	122.0(4)	118.8(8)	117.2(15)
Distance out of plane of the metal (Å)*	-0.040(14)	0.044(10)	-0.555(13)	-0.545(3)	-0.553(5)

* mean plane comprised of atoms C1, N1, N2, C2, C3, N3, N4 and C4

geometry is squared pyramidal with the metal atoms lying around 0.555 Å outside of the mean plane comprised of atoms C1, N1, N2, C2, C3, N3, N4 and C4 (Figure 5 and Table 2). The metal atom is coordinated to the dideprotonated tetradentate bis(thiosemicarbazone) and one chloride or solvent molecule occupies the apex position. For the indium complex, the coordination mode of the ligand leads to the formation of three five-member chelate rings that confer high stability to the complex as for the Cu and Ni complexes. However, for the Ga and Zn complexes the ligand coordinates in a tetradentate mode through the N1, N3, S1 and S2 atoms, leading to the formation of three, four and six member chelate rings, being the dihedral angles N2-C2-C3 and C2-C3-N3 different (Table 2). Due to this coordination mode the square pyramidal geometry of the Zn and Ga complexes is more distorted. All the C2–C3, C2–N2 and C3–N3 bond distances are within the range of other complexes found in the literature, which depend on the nature of the metal center, and do not deserve further comments. The distances of the bonds (Table 2) are in the same range for all complexes, which indicates the versatility of the TSC to accommodate different sizes and preferences coordinative metal ions, despite the higher rigidity of the backbone in comparison with other TSC due to the acenaphthenequinone group.

Although ^{64}Cu is a well-known and used metal in PET studies and it has been the metal of choice for the study of ATSM, the generation process is not optimal for clinical applications as it can only be obtained from a cyclotron despite its very favorable half-life ($t_{1/2}$) of 12.7 h which allows for transport to remote sites and



Scheme 4 Coordination equilibrium found in bis(4-allyl-3-thiosemicarbazonato) metal complexes

further derivatizations. Due to the availability of generators, the research in ^{68}Ga aqueous chemistry has strongly arisen in the past decade within the radiochemistry and molecular imaging research community, which is now geared towards pre-clinical as well as clinical applications of this radionuclide. The generation process of ^{68}Ga is more straightforward and affordable for clinical application due to the use of a ^{68}Ge/^{68}Ga generator that allows obtaining a readily available dose in a shorter time and can be done in the clinical facility. Furthermore, ^{68}Ga $t_{1/2}$ is 68 minutes which is in the desired range for an imaging experiment. The chemical process where the hot derivative is involved has to be rapid enough but timing is not a crucial issue as with ^{18}F which has a $t_{1/2}$ of 109.7 min and significantly more challenging multi-step radiochemistry in our experience. It also, allows the patient to receive a lower radiation dose post injection and after the experiment.[70] Considering these advantages and the appearance of new radionuclides for clinical uses, the preparation of bis(thiosemicarbazonato) acenaphthenequinone complexes of gallium(III) and indium(III) was studied.[3] The cold complexes of Ga(III) and In(III) with ATSM and acenaphthene backbones were synthesized following the described procedures in a template process from the Zn complex in the case of acenaphthenequinone and from the ligand for ATSM. The side groups included in this study were methyl and allyl for the acenaphthenequinone derivatives. The optical imaging of the allyl functionalized zinc(II), gallium(III) and indium(III) complexes was studied in PC-3 and HeLa cells by 1 and 2-photon fluorescence imaging as well as by FLIM (Figure 6). The possibility of obtaining 2-photon fluorescence lifetime imaging maps allows to ascertain that the complexes are intact in the imaging time scale. In addition, the radiolabeling experiments were carried out by transmetalation of "cold" Zn(II) precursors to "hot" ^{68}Ga and ^{111}In derivatives and studied by radio HPLC (Figure 7). Challenges of the complexes

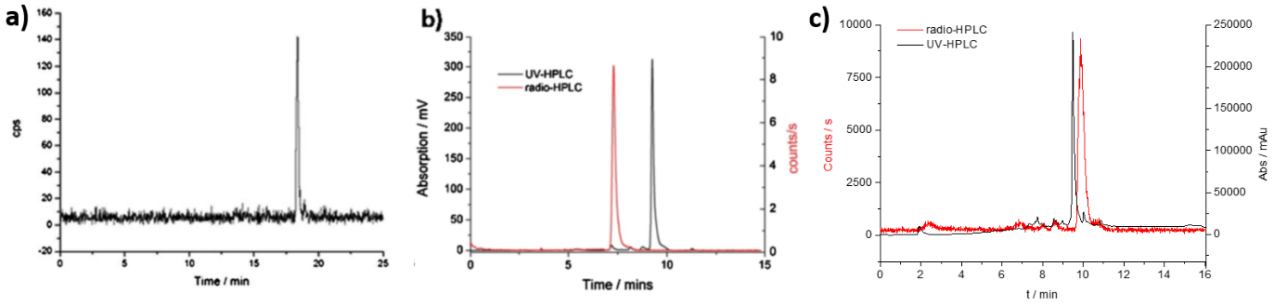


Figure 7 HPLC traces from different radiolabelling experiments: a) Radio-HPLC trace of the ^{64}Cu labelling for the bis(4-ethyl-3-thiosemicarbazonato) acenaphthenequinone complex[2]; b) overlay of UV-HPLC trace for the Zn(II) bis(4-allyl-3-thiosemicarbazonato) acenaphthenequinone precursor and the radio-HPLC for the ^{111}In labelling[3]; c) overlay of the UV-HPLC and radio-HPLC traces for the ^{68}Ga microwave labelling of the bis(4-allyl-3-thiosemicarbazonato) acenaphthenequinone. b) c) Adapted from Ref. [1] with permission from The Royal Society of Chemistry.

in water and serum revealed that both 'cold' precursors and 'hot' derivatives of ^{68}GaATSM and ^{111}InATSM have an extremely low kinetic stability in aqueous environments compared to ^{64}CuATSM and were, therefore, not appropriate for radiolabeling studies thus far.

Imaging of hypoxia

Hypoxia and Cancer

In the 1950s, Gray and colleagues first pointed out both the presence of low oxygen tensions in human tumors[71] and the possibility that radiation results can be influenced by those regions.[71],[72] Therefore, research was focused on allowing for an increase in the sensitivity of the hypoxic cells by using either hyperbaric oxygen chambers[73] (before early 1970s) or small molecules mimicking oxygen's effects[74] (after 1980s). By the 1990s, it was known that the concentration of oxygen levels varies in human tumors.[75] To date, variety of studies have confirmed the importance of tumor hypoxia and underlined its detrimental role in tumor progression and treatment. Therefore, extensive studies have been conducted in order to decipher, limit and trace hypoxic conditions pathology.

In tumors, the neoplastic cells often consume more oxygen than they are supplied, resulting in growing low oxygen level tissue areas. Neoplastic hypoxia can be caused either by perfusion-limited oxygen delivery (acute hypoxia) which results from structural and functional abnormalities of tumor microvessels; by diffusion-limited oxygen delivery (chronic hypoxia) as a result of the increased distance between the cell and the blood vessel; or from tumor-associated and/or therapy induced anemia leading to reduced oxygen capacity in blood (anemic hypoxia).[76],[77] In hypoxic cells, protein synthesis is reduced leading to restrained proliferation and cell death. On the contrary, diversification of cell cycle distribution is observed as a consequence of prolonged exposure in hypoxic conditions. Those changes are common causes of tumor progression as tumor cells establish mechanisms in order to evade the hostile environment generated by hypoxia. In addition, hypoxia is highly related to apoptosis of both normal and neoplastic cells[77-79] and leads to therapy resistance through various pathogenic mechanisms (selection of potentially p-53 mediated senseless cells[78],[79]; (ii) inadequate exposure to anticancer drugs through the aforementioned increase in distance between cells and blood vessels (acute hypoxia)[80]; and (iii) via the upregulation of drug resistant genes and "encoding genes"[81]). Alterations in gene expression with subsequent changes in the proteome and/or the genome[78] and cell selection[78],[79],[82] are the underlying mechanisms of hypoxia's interference with tumor malignancy. Tumor hypoxia is considered as a valuable target for tumor prognosis because of its relationship with malignant progression, combined with the development of resistance to cancer therapies. As a result, a variety of detection techniques have been developed throughout the years in order to distinguish hypoxia in tumors. This include the identification of low oxygen areas[83],[84] the targeting of endogenous markers, such as HIF-1 and CAIX,[84],[85] the employment of exogenous hypoxia markers, such as 2-nitroimidazoles,[86] and the use of non-invasive techniques such as PET imaging and MRI.[24],[87],[88]

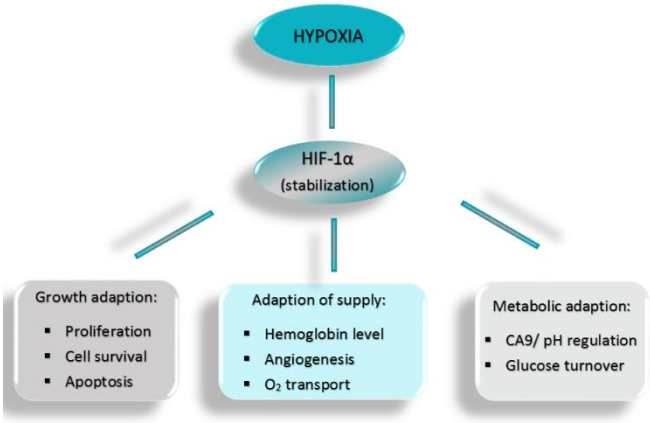


Figure 8 Effects of hypoxia to the organism regulated by HIF

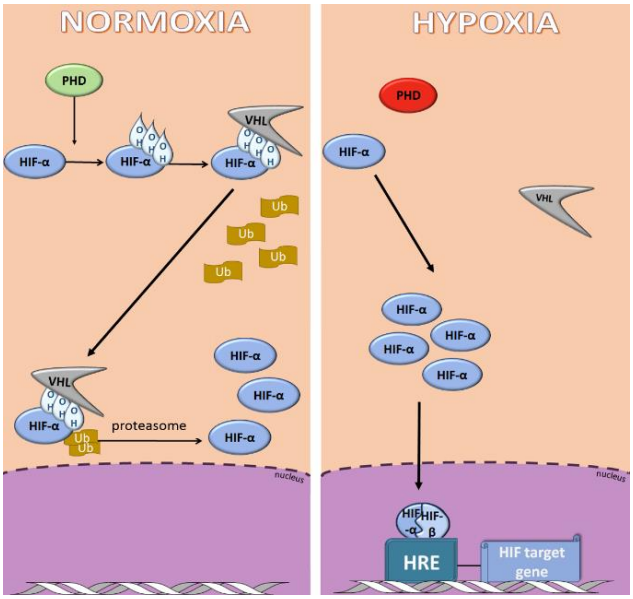


Figure 9 Mechanism of targeted genes activation by HIF under normoxia (left) and hypoxia (right)

Hypoxia inducible factors (HIF)

Hypoxia inducible factors (HIFs) are a family of transcription factors notably sensitive to oxygen gradients, pO_2. Such transcription factors are capable of activating complex gene expression models directed to adapt into low oxygen conditions. The HIF family consist of 3 different members (HIF-1, HIF-2 and HIF-3), all of them constituted of two subunits (α and β). The alpha subunit contains an oxygen dependent degradation domain (ODD) resulting from the uniqueness of each gene and is responsible for the regulation of the hypoxia inducible behavior (Figure 8). The beta subunit is a hydrocarbon nuclear translocator domain (ARNT) ubiquitously expressed instead.[89],[90] Tumor hypoxia is mainly related with HIF-1α and HIF-2α factors and a variety of alteration in gene regulation have been proposed (Figure 9). Although they similarly activate the gene transcription triggered by hypoxia; HIF-2α is mainly active in conditions of prolonged hypoxia, while HIF-1α is active in acute hypoxia.[90],[91]

Since 1993, it has been demonstrated that iron chelators could induce HIF-1α.[92] Later investigations have evidenced that a variety of metal (Zn(II), Cu(II), Ni(II), Co(II)) could mimic this iron's characteristic by stabilizing HIF-1α.[93] This could potentially lead to the development of metal chelators which will reduce HIF's expression.

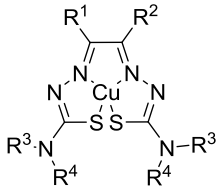
Imaging of Hypoxia with ATSM complexes

The imaging of hypoxia has been based for a long time in the use of nitroimidazole compounds labeled with ^{18}F or ^{123}I, being the most used ^{18}FMISO, which in a reductive environment gets reduced and trapped in hypoxic cells and tissues.[94]

However, nitroimidazoles present a number of disadvantages for their routine use as low lipophilicity, slow pharmacokinetics, low tumor-to-muscle ratios or the limited clearance from normoxic tissue.[95],[96]

Bis(thiosemicarbazonato) complexes for the imaging of hypoxia were first employed by Fujibayashi *et al.* in 1997 in a rat heart ischemic model.[14] The retention of a ^{62}CuATSM derivative was found to be around 80% under hypoxic conditions in perfused isolated hearts while under normoxic conditions the retention was not higher than 20%. The hypoxic selectivity of CuATSM *in vitro* and *in vivo* was later confirmed by other groups.[15],[16] and related to the pO_2 in tumors.[97]

Since the appearance of this work, bis(thiosemicarbazonato) complexes derived from aliphatic 1,2-dicarbonylic compounds have attracted the interest of many research groups worldwide. The modification of the backbone and substituents of the thiosemicarbazides was rapidly explored (Figure 10) and the



	R^1	R^2	R^3	R^4
GTS	H	H	H	H
GTSM	H	H	CH_3	H
PTS	CH_3	H	H	H
PTSM	CH_3	H	CH_3	H
$PTSM_2$	CH_3	H	CH_3	CH_3
PTSE	CH_3	H	C_2H_5	H
PTSP	CH_3	H	C_6H_5	H
ATS	CH_3	CH_3	H	H
ATSM	CH_3	CH_3	CH_3	H
CTS	C_2H_5	CH_3	H	H
CTSM	C_2H_5	CH_3	CH_3	H
DTS	C_2H_5	C_2H_5	H	H
DTSM	C_2H_5	C_2H_5	CH_3	H
DAGTS	NH_2	NH_2	H	H
MAGTS	Me	NH_2	H	H

Figure 10 Structures of copper bis(thiosemicarbazonato) complexes studied during the investigation of the properties and its mechanism of hypoxia selectivity

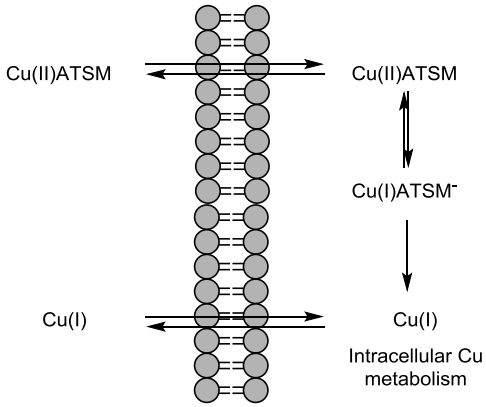


Figure 11 Proposed uptake mechanism of CuATSM in a cellular environment under hypoxia

hypoxic retention *in vitro* evaluated.[16] The variation on the hypoxic selectivity in cells was related mainly to the lipophilicity and redox potential. These parameters were tuned in an attempt to obtain an optimal response.[98]

The mechanism of action of CuATSM as hypoxia tracer has been largely debated together with its behavior as a copper transporter *in vitro* and *in vivo*. The first proposed mechanism of action of copper BTSC relies on the fact that the complex crosses the membrane due to its high lipophilicity and gets trapped in the cell under hypoxic condition with the resulting reduction of Cu(II) to Cu(I).[14] This mechanism was further elaborated by suggesting that CuATSM is reduced in all cellular environments, not only under hypoxia, resulting in a Cu(II)ATSM and $Cu(I)ATSM^-$ equilibrium. However, in the absence of oxygen, the unstable $Cu(I)ATSM^-$ complex cannot be reoxidized to the stable Cu(II) species and will protonate and dissociate in the presence of copper (Figure 11).[18],[99] Density Functional Theory (DFT) studies, electrochemical measurements and UV-vis spectra have supported this hypothesis.

In an attempt to elucidate the location of the intracellular reduction, Fujibayashi *et al.* analyzed by HPLC lysed Ehrlich tumor cells.[100] The reduction process was found to happen mainly in the microsome/cytosol instead than the mitochondria, to be heat sensitive and enhanced by the addition of NADPH, fact that points to an enzymatic reduction of CuATSM.

The mechanism of cellular stability and related decomposition pathways of Cu(II) bis(thiosemicarbazones) has also been investigated by using DFT calculations.[101],[102] In these work it was revealed that the HOMO-LUMO gap of the complex was surprisingly small (in the order of 0.05 eV) compared to other transition metal complexes and dependent on the substituents. The importance of the methyl groups in the ATSM backbone was also demonstrated. Derivatives without these groups would undergo protonation and degradation intracellularly hence not being hypoxic selective.[99],[102]

Furthermore, the characteristics of the series in Figure 10 were explored and compared by UV-vis spectroscopy, cyclic voltammetry and DFT (looking at structures, vibrations, protonation and reduction). These results suggested an

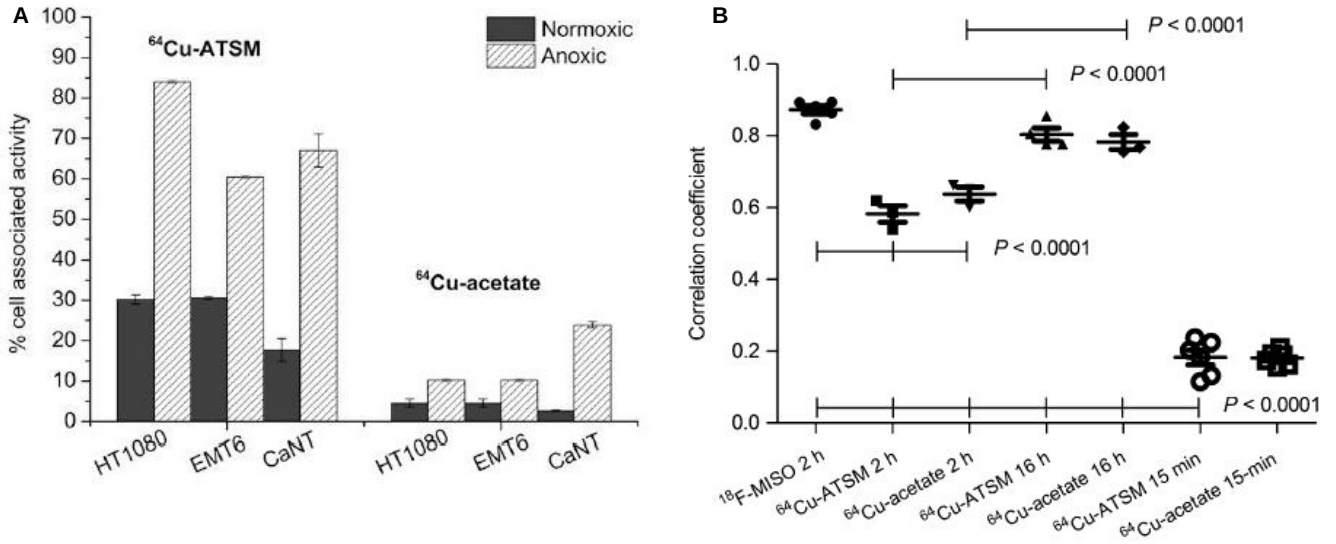


Figure 12 A Graph showing the radioactivity retention *in vitro* of ^{64}CuATSM and ^{64}Cu(OAc)$_2$ at 30 min after incubation in HT1080, EMT6 and CaNT cell lines. (CaNT cells were excised from *in vivo* tumors as they cannot be cultured *in vitro*); B. Graph showing the correlation coefficients accounting for the spatial correlation between autoradiography and EF5 immunofluorescence of tumors excised at different times after the administration of ^{18}FMISO, ^{64}CuATSM and ^{64}Cu(OAc)$_2$. (This research was originally published in JNM. R. Hueting, *et al.* A Comparison of the Behavior of ^{64}Cu-Acetate and ^{64}Cu-ATSM *in vitro* and *in vivo*. J. Nucl. Med. **2014**, 55, 128-134. © by the Society of Nuclear Medicine and Molecular Imaging, Inc.)

irreversible reduction of CuPTSM and CuPTS (where PTS is pyruvaldehyde bis(thiosemicarbazone) in the presence of an acid. However, UV-vis and Cyclic Voltammetry (CV) studies proved that CuATSM undergoes a reversible one electron reduction. Despite the approximations made during the calculations, DFT works permitted to extract trends between the redox potential of the complexes, the LUMO energy and the alkyl groups present in the backbone. Such calculations proposed that the HOMO does not vary through the series while the LUMO varied from CuATSM, where it is metal based generating a singlet state in reduced species to CuPTS, where it is ligand based originating a triplet state.[103] It was demonstrated that both compounds were reduced in the conditions found *in vitro.* However, CuPTS was reduced more efficiently than CuATSM. CuPTS is effectively trapped into the cell as, protonated and dissociated, releases Cu(I) cations capable to bind intracellular proteins. Under the same circumstances, CuATSM can be reoxidized and the original Cu(II) species can escape from the cell by diffusion.

The one-electron reduction potentials and absolute pKa values for a list of complexes, depicted in Figure 10, were also studied by DFT. The low values obtained for the pKa of all complexes demonstrated that the protonation in the pH of normal tissue is avoided and the complexes can cross cellular membranes. Also, electron donating groups at R1 and R2, as NH_2, tend to destabilize the complex by increasing the energy of the LUMO. The cationic species Cu(I)ATSMH_2^+ was proposed as the initial compound trapped in hypoxic cells.[101]

However, the results of a retention study of ^{64}CuATSM in different cell lines showed contradictory results that did not follow completely the above mentioned mechanism. Therefore, it was proposed that ^{64}Cu was not only bound to the ATSM ligand but after dissociation, the copper was added into the intracellular pool. For this reason, radioactive copper would join the intricate copper metabolism where Cu(I) would be protected from reoxidation and be transported out of the cell by an active mechanism.[104]
The difference in retention in the different cell lines tested was attributed to the presence and abundance of specific Cu(I) transporters. Measured cellular levels can be equivalent to the active transport of ^{64}Cu rather than the uptake of CuATSM so the results should be taken with caution.

There have also been inconsistencies in prostate cancer models. Lewis *et al.* observed that the retention of ^{64}CuATSM under hypoxia in different prostate cancer cell lines was significantly lower when compared to other cancer cell lines.[105] It was attributed to a defense mechanism where the redox equilibrium of the cell is altered and intracellular reductants as NADPH are consumed by the over expression of the Fatty Acid Synthase (FAS) hence making difficult the reduction of Cu(II) to Cu(I). This was demonstrated by blocking the FAS that produced the expected increase in retention of ^{64}CuATSM *in vitro* in four different prostate cancer cell lines.

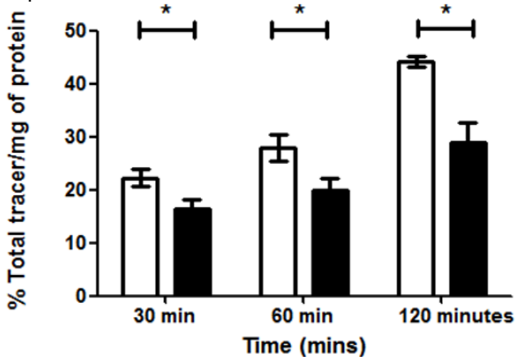


Figure 13. [^{68}Ga](bis(4-allyl-3-thiosemicarbazonato) acenaphtenequinone uptake in EMT6 cells under hypoxic (white bars) or normoxic conditions (black bars). Hypoxia in cells was induced for 20 min prior to addition of the tracer in 1% O_2 and cells were maintained under the same conditions for the remainder of the time course. Reproduced from Ref. [1] with permission from The Royal Society of Chemistry.

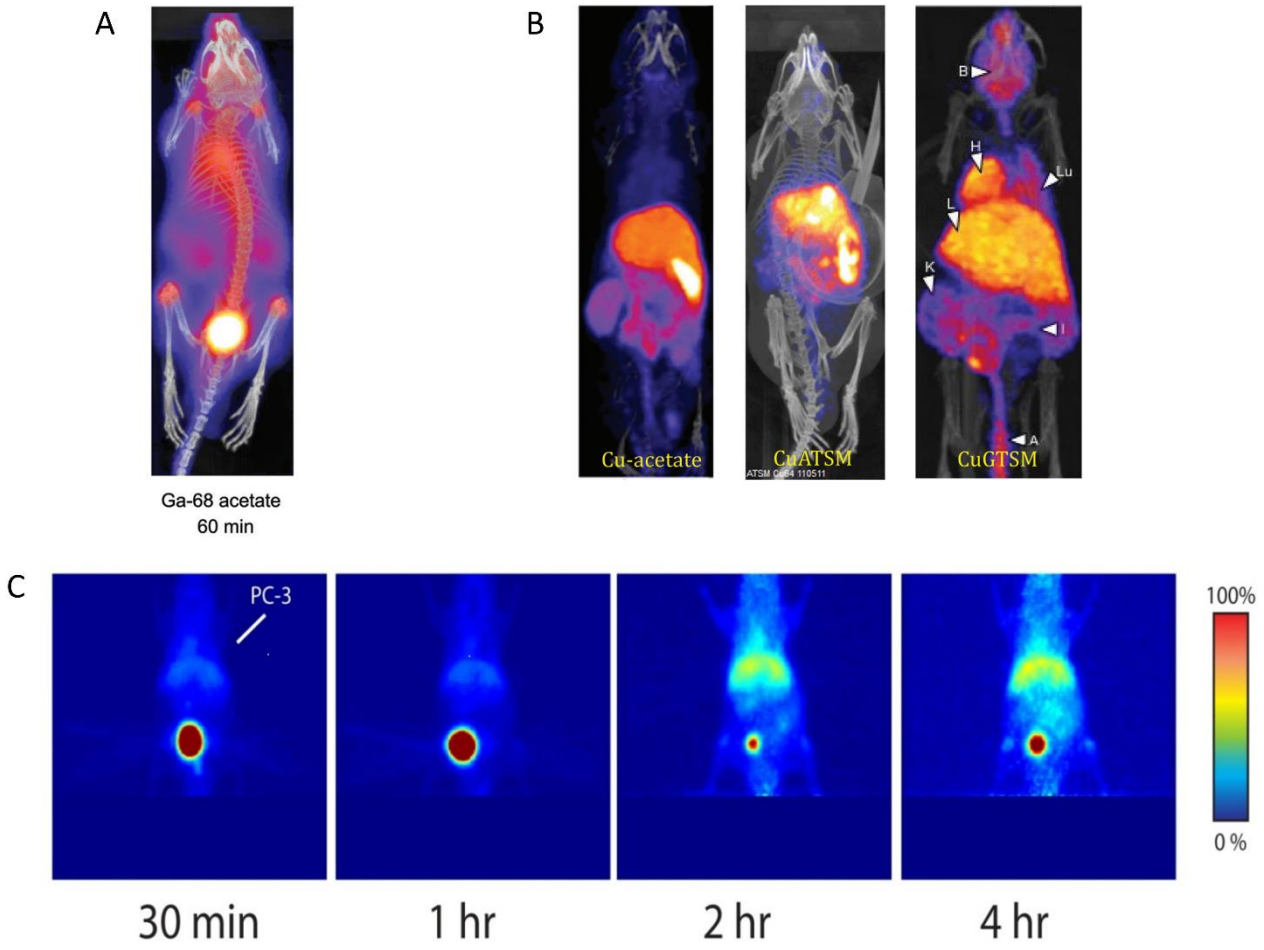


Figure 14 *In* vivo imaging experiments with metal acetates and corresponding complexes of bis(thiosemicarbazones). (A) ^{68}Ga acetate only 60 min post injecion[113]; (B) Images of ^{64}Cu acetate, ^{64}CuATSM and ^{64}CuGTSM 30 min post injection. It can be seen how copper acetate shows a similar biodistribution to CuATSM with rapid liver uptake. CuGTSM shows uptake not only in the liver and gut but also in the brain and the myocardium[113]; C) MicroPET images of [^{68}Ga]bis(4-ethyl-3-thiosemicarbazonato) acenaphthenequinone complex in nude mice at different time points.[1] A) B) Reproduced from Ref. [113] with permission from The Royal Society of Chemistry; C) Reproduced from Ref. [1] with permission from The Royal Society of Chemistry

The latest contributions to the understanding of the fate of bis(thiosemicarbazonato) complexes *in vitro* and *in vivo* were made by Dilworth *et al.* in 2013 and 2014. In the first work,[4] GTS (glyoxal bis(4-ethyl-3-thiosemicarbazone)) and ATSM metal complexes were synthesized with a BODIPY fluorophore attached to them in order to overcome the quenching of the fluorescence in the paramagnetic copper(II) complexes and enhance it in the rest. The lifetime of the complexes was studied to evaluate their stability and speciation *in vitro*. The integrity of different BODIPY-tagged ATSM-like complexes in PC-3 and HeLa cells was evaluated by FLIM. The poor fluorescence of the ligands was used as an indicator of the presence of intact complex in cells by confocal microscopy. Furthermore, the stability of the metal complexes *in vitro* was assessed by acquiring lifetime imaging maps and decay curves (Figure 4, bottom) under 2-photon excitation. Then, the lifetime was calculated in HeLa cells and in aerobic conditions for times up to 1 h by FLIM coupled to confocal laser scanning microscopy (Figure 4, bottom). The data confirmed that the copper(II) and nickel(II) BODIPY-tagged ATSM-like complexes remain unchanged in cells for times up to 1 h while the zinc(II) derivative did not provide conclusive data. When compared to the latter species, the copper(II) BODIPY-tagged derivative without the methyl groups in the backbone was found to be completely dissociated in 20 min.

The second work[106] compared the behavior of $^{64}Cu(OAc)_2$ and ^{64}CuATSM *in vivo*. While $^{64}Cu(OAc)_2$ was assumed to be used as a control as it showed minimal uptake in EMT6 cells in previous works,[52] it was found that the biodistribution was surprisingly similar as when ^{64}CuATSM was used. The autoradiography of tumors compared to EF5 inmunohistochemical staining revealed that there was no spatial correlation for $^{64}Cu(OAc)_2$ or ^{64}CuATSM under hypoxia after 15 minutes or 2 h in contrast to ^{18}FMISO. However, the correlation improved at longer times for ^{64}CuATSM but also for $^{64}Cu(OAc)_2$ (Figure 12). These data suggest that a proportion of radiocopper, in the ATSM case, is ^{64}Cu bound to proteins and not intact complex revealing that copper metabolism is being tracked. The hypoxia selectivity seems to be determined by the cellular processing of copper that is released from the thiosemicarbazone via a redox process. This process is necessary to obtain free copper in cells but does not control the selective uptake. Ultimately, the data suggests that the relationship between CuATSM and hypoxia is not entirely direct *in vivo* compared to *in vitro*.

The application of bis(thiosemicarbazonato) complexes has not been limited just to the imaging of hypoxic regions in tumors, it has also been used in the imaging of ischemic and hypoxic myocardium.[94],[107] In this field, the modification of the complex to be more sensitive towards cardiac hypoxia has recently been explored.[108]

Copper bis(thiosemicarbazones) based on aliphatic cores have proved very useful in delimiting hypoxia areas in tumors and myocardium. The functionalization with targeting molecules is possible and does not seem to alter greatly the hypoxic selectivity providing opportunity to obtain targeted delivery and therapy probes. The use of ^{64}CuATSM in therapy has revealed to provide improved cytotoxicity and DNA damage under hypoxia.[109] However, as some results have highlighted CuATSM undergoes a complex uptake mechanism *in vitro* and *in vivo* and its application for the imaging of tumors seems to be not universal. Regarding the therapeutic applications, the optimization of the common ^{64}CuATSM needs to be undertaken. Therefore, care should be taken in its application and study of the results.

Interestingly, the nature of the bis(4-allyl-3-thiosemicarbazone) acenaphthenequinone gallium(III) complex was studied as further assays revealed that it shows hypoxic selective behavior under the conditions tested in a range of cells. In this work, the improved synthesis of mono and bis(thiosemicarbazone) ligands and hot complexes was investigated using microwave synthesis. In the case of the ligands, the yields were comparable to the ones obtained by conventional for the mono(thiosemicarbazones) and improved in the case of the bis(thiosemicarbazones). Furthermore, the reaction time was reduced from 2 h to under 10 min. In the case of the hot complexes, the microwave synthesis helped in reducing the reaction time, a crucial factor when using radionuclides.[1]

Based on the proposed mechanism of hypoxia selectivity described above, the redox potentials of the metal complexes must be within a very specific range. According to this mechanism, the behavior of CuATSM is believed to be ruled by the redox chemistry that favors the release of copper in vitro but does not regulate the uptake. However, in the case of gallium, no redox chemistry is possible under biological conditions so no hypoxic behavior was expected. Surprisingly, the [^{68}Ga]bis(4-allyl-3-thiosemicarbazonato) acenaphthenequinone complex was hypoxia selective as evaluated by using flow cytometry and radioactive cell count (Figure 13). The electrochemistry of the gallium complex and similar non-copper derivatives was studied to evaluate if there was an influence of the backbone but no reduction or oxidation waves were observed. The proposed hypothesis is that gallium(III), which shares certain characteristics with iron(III) as charge or size, an ionic radius of 62 pm compared to 55 pm in iron(III)[110], is released intracellularly and then trapped by one of the potent iron chelators present in the cells. So, in this case the hypoxic behavior could be attributed to the track in changes in the iron metabolism under hypoxia which is known to be altered.[111],[112]

Preliminary *in vivo* studies had also been conducted in mice with a ^{68}Ga-labeled bis(4-ethyl-3-thiosemicarbazonato) acenaphthenequinone complex (Figure 14). The results revealed that the compound accumulated in the liver after injection to be distributed over the the body of the mouse with time. A concentration in the lungs could be observed 2 and 4 h post injection.

Conclusions

Bis(thiosemicarbazone) ligands and their metallic complexes have been extensively investigated over the last 50 years due to their pharmacological activity and potential in cancer theranostics applications. The family of CuATSM have perhaps been the most effective class of bis(thiosemicarbazonato) metal complexes in the field of imaging diagnostics in hypoxic tumors. CuATSM provided a brand new class of compounds that allowed to delineate the origin and extent of hypoxia in tumors and heart diseases. The mechanism by which CuATSM-like complexes are hypoxia selective involves intricate intracellular reduction-oxidation events leading to an abundance of Cu(I) species. Indeed, in hypoxic environments, these redox equilibria favor the formation of Cu(I) containing species and their consequent dissociation in a biological environment. A variety of transition metals (Zn(II), Ga(III), In(III), Ni(II)) were also investigated providing important results that elucidated the fate of such redox related processes in cells under hypoxic regime.

The applicability of bis(thiosemicarbazones) as multimodal bioimaging probes was further explored by redesigning the ATSM ligands. By incorporating an acenaphthenequinone moiety within the bis(thiosemicarbazone) backbone, novel luminescent species were achieved and described in this work. Such aromatic metal complexes could potentially open a new prospective in the use of bis(thiosemicarbazones) as multimodal imaging tools. These systems can be used *in vitro* for optical imaging techniques (fluorescence microscopy) and *in vivo* for PET or SPECT imaging. The employment of a range of radionuclides such as ^{64}Cu, ^{68}Ga or ^{111}In were also explored and the most representative examples presented in this review.

Bis(thiosemicarbazonato) metal complexes certainly have the potential to catalyze the search for the ideal bifunctional chelator and the radiotracer design and testing research in multimodality imaging. Considering the interest that theranostics are currently generating in the scientific community, it is assured that in future the study of novel bis(thiosemicarbazonato) metal complexes will continue in order to discover new and more efficient hypoxia selective anti-tumor agents.

Acknowledgements

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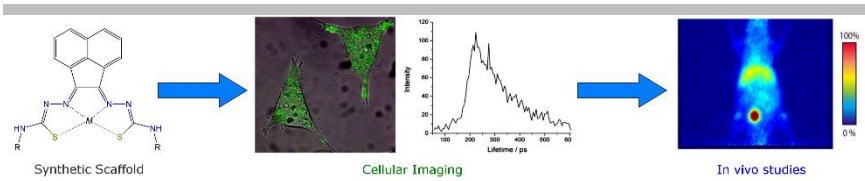
Layout 2:

PERSONAL ACCOUNT



The aim of this review is to provide a personal account of the general background on the use of bis(thiosemicarbazonato) metal complexes in molecular imaging, which is central to the recent work in this field. We have a long-lasting interest in understanding the structure-function relationships underlining the potential of bis(thiosemicarbazonato) metal complexes for applications in multimodal imaging involving PET/SPECT/optical imaging modalities. The molecular design and the selectivity of this class of compounds for the evaluation of hypoxia is also discussed herein.

*Fernando Cortezon-Tamarit, Sophia Sarpaki, David G. Calatayud, Vincenzo Mirabello and Sofia I. Pascu*****Page No. – Page No.***

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